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Inhibitory Effects of Acyclic Retinoid (Polyprenoic Acid) and Its Hydroxy Derivative on Cell Growth and on Secretion of α-Fetoprotein in Human Hepatoma-derived Cell Line (PLC/PRF/5)

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Acyclic retinoid (polyprenoic acid) has a slightly different structure from retinoic acid. However, acyclic retinoid acts similarly to retinoic acid, because both bind to cellular retinoic acid-binding protein and cellular retinoid-binding protein, F-type, with the same strong binding affinity. We studied the effects of acyclic retinoid, the 7-hydroxy derivative of acyclic retinoid (7OH-acyclic retinoid) and retinoic acid on a human hepatoma-derived cell line PLC/PRF/5 (Alexander cells). Acyclic retinoid inhibited cell growth with an ID_{50} value of 14 μM, and reduced cell viability with an LD_{50} value of 86 μM. The ratios of LD_{50} value to ID_{50} value were 6.1 for acyclic retinoid, 2.4 for 7OH-acyclic retinoid and 1.4 for all-*trans*-retinoic acid. Taking this ratio as a parameter of relative cytotoxicity, we concluded that acyclic retinoid is the least toxic compound. Growth inhibition of cells by acyclic retinoid was associated with the incorporation of ^{3}H-thymidine in the logarithmic phase. Acyclic retinoid reduced secretion of α-fetoprotein (AFP) and reciprocally increased secretion of albumin in the culture media, suggesting that acyclic retinoid influences gene expression of these proteins. Thus, acyclic retinoid, one of the less toxic retinoids, inhibits cell growth of human cancer cell line PLC/PRF/5 and appears to alter gene expression of AFP and albumin toward a "normal" direction.

Key words: Acyclic retinoid — Human hepatoma cell line (PLC/PRF/5) — α-Fetoprotein — Albumin

Retinoids are well documented to inhibit tumor development in animal experiments and clinical applications,[1] and attempts have made to develop new retinoid-based preventive agents for human hepatoma.[2–7] We have studied the effects of acyclic retinoid (polyprenoic acid) on human hepatoma-derived cell line PLC/PRF/5 (Alexander cells). Acyclic retinoid is a synthetic derivative of polyprenoic acid and was positive in screening by means of binding assay to cellular retinoic acid-binding protein (CRABP) and cellular retinoid-binding protein, F-type (CRBP(F)).[8] Although the structure of acyclic retinoid is slightly different from that of all-*trans*-retinoic acid, acyclic retinoid bound to CRABP and CRBP(F) as strongly as did all-*trans*-retinoic acid and the trimethylmethoxyphenyl analog of retinoic acid ethylester (TMMP).[8] It seems likely that acyclic retinoid acts on cells through the same pathway as that of retinoic acid. We have reported that oral application of acyclic retinoid significantly reduced the incidence of skin tumors induced by 7,12-dimethylbenz[*a*]anthracene plus croton oil on mouse skin, as well as the tumor incidence of "spontaneous" hepatoma-bearing mice (C3H/HeNCrj).[2] In the experiments with acyclic retinoid and TMMP on inhibition of hepatoma induced by 3′-methyl-N,N-dimethyl-4-aminoazobenzene in rats, the acyclic retinoid diet (40 mg/kg/day) did not cause any reduction in the increase of body weights of rats compared with that of the control, whereas the TMMP diet (10 mg/kg/day) caused toxic effects.[2] From these results, it appeared that acyclic retinoid is less toxic than TMMP, although they have the same inhibitory potencies on the tumorigenesis.

The effects of acyclic retinoid on PLC/PRF/5 cells were compared with those of retinoic acid with regard to cell growth, cytotoxicity, secretion of α-fetoprotein (AFP) and synthesis of albumin. Acyclic retinoid inhibited the synthesis/secretion of AFP earlier in time as compared to its action on cell growth, and induced a concomitant increase of albumin secretion. The results suggest that acyclic retinoid might be useful in the prevention of human hepatoma.

MATERIALS AND METHODS

Chemicals Acyclic retinoids (polyprenoic acid or open-chain C_{20} analog of retinoic acid; all *trans*-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid (E5166)) and its 7-OH derivative (Fig. 1) were kindly provided by Eisai Co., Tokyo. All-*trans*-retinoic acid was purchased from Sigma Chemical Co., St. Louis, Mo.

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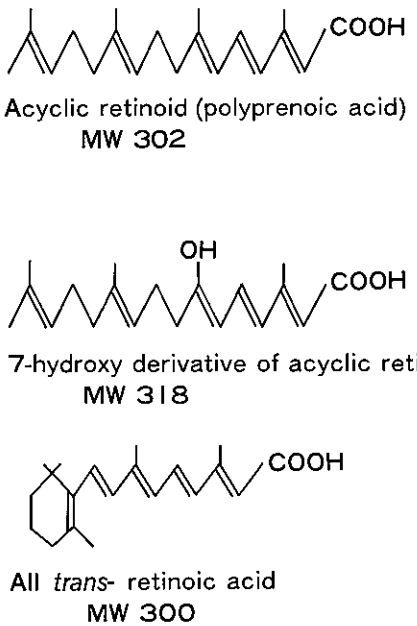

Fig. 1. Chemical structures of acyclic retinoid (E5166), 7-OH derivative of acyclic retinoid and all-*trans*-retinoic acid.

Cell culture Human hepatoma-derived PLC/PRF/5 cell line (Alexander cells)[9] were maintained in Eagle's minimum essential medium (MEM) (Osaka Microbiology Laboratory, Osaka) supplemented with 10% fetal calf serum (Hyclone Laboratory Inc., Utah) and 50 μg/ml kanamycin (Flow Laboratories, Irvine, UK) in an atmosphere containing 5% CO_2 at 37°C. Culture medium was changed every week, and the cell population was adjusted to 4×10^4 cells/ml at each medium change. For the S phase synchronous culture, cells growing in the logarithmic phase were first incubated in MEM with 10% FCS and 50 m*M* thymidine (Kohjin Co., Ltd., Tokyo) for 24 h, followed successively by incubation in MEM alone for 10 h and in MEM containing 20 m*M* hydroxyurea (Nakarai Chemicals Ltd., Kyoto) for 16 h. After serial treatments as mentioned above, the cells, which should be at the G_1-S phase border (time 0), were subjected to further experiments.

Treatment of cells with retinoids Retinoids, dissolved in dimethyl sulfoxide, were added to culture media to give the final concentration of 0, 0.1, 0.5, 1, 5, 10, 20, 40, 80, or 100 μM. Cells were exposed to retinoids for up to 6 days, and total cell numbers as well as cellular viabilities were examined on experimental days 0, 1, 2, 4, and 6 using Bürker-Türk counting plates for the former and the trypan blue dye exclusion test for the latter.

Determinations of AFP and albumin in culture media Culture media taken on experimental days 0, 1, 2, 4, and 6 were centrifuged at 7,600*g* for 15 min and the supernatants were subjected to determination of AFP and albumin. AFP was measured by a radioimmunoassay using an AFP RIA kit (Novo Laboratories Inc., Tokyo). Albumin was determined by ELISA using rabbit anti-human albumin Fab′ fragment conjugated with horseradish peroxidase (Cosmo Bio Co., Tokyo).

Incorporation of ^{3}H-thymidine into the cells in S-phase synchronous culture Cells at time 0 (h) in the synchronous culture were initiated to proceed to the S-phase by replacing the culture media containing hydroxyurea with prewarmed MEM supplemented with 0, 1, or 10 μM acyclic retinoid, and were incubated for up to 14 h. At time 0, 2, 4, 6, 8, 10, 12 or 14 h, cells were exposed to ^{3}H-thymidine (4.77 TBq/mmol, Amersham Japan Co., Tokyo; final concentration 5×10^{-5} *M*) for 30 min, and radioactivity incorporated into the cells was counted in a liquid scintillation counter LS 8500 (Beckman, USA).

Statistical analysis Experiments were carried out three times, one of which was in triplicate. Differences among median values were statistically analyzed by applying the Mann-Whitney-Wilcoxon test.

RESULTS

Effects of retinoids on cell growth rates and viability PLC/PRF/5 cells were treated with various concentrations of three retinoids, acyclic retinoid, 7OH-acyclic retinoid and retinoic acid, for 6 days. Figure 2a shows the number of cells 6 days after treatment. All three retinoids showed inhibitory effects on cell growth from 10 μM and completely inhibited the growth at 100 μM. The ID_{50} values (50% inhibition of cell growth) were 14 μM for acyclic retinoid, 26 μM for 7OH-acyclic retinoid and 28 μM for all-*trans*-retinoic acid. The ID_{50} value of acyclic retinoid was approximately one-half of that of all-*trans*-retinoic acid, and the difference was statistically highly significant ($P<0.01$). Figure 2b shows the viability of cells 6 days after treatment, which was determined as the percentage of living cells with respect to the total number of cells. In contrast to the inhibition of cell growth, the three retinoids did not have any effect on viability at concentrations of up to 20 μM. The LD_{50} values were 86 μM for acyclic retinoid, 61 μM for 7OH-acyclic retinoid and 40 μM for retinoic acid. When the LD_{50} values were compared with the ID_{50} values, reduction of viability by the three retinoids appeared at higher concentrations than inhibition of cell growth. If the concentration ratio for reduction of viability to inhibition of cell growth reflects the toxic effects of retinoids, acyclic retinoid is less toxic to the cells than 7OH-acyclic retinoid and all-*trans*-retinoic acid (Table I). We further examined the toxicity of acyclic retinoid to PLC/PRF/5 cells. Figure 3 shows growth curves of cells treated with 10 μM acyclic

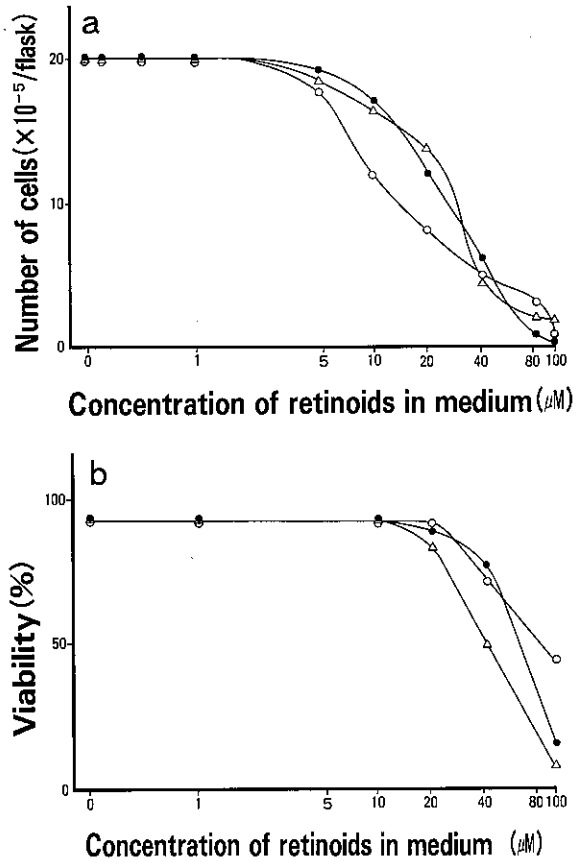

Fig. 2. (a) Inhibitory effects of retinoids on the growth of PLC/PRF/5 cells. The cells were treated with retinoid for 6 days. (b) Viabilities of PLC/PRF/5 cells after exposure to retinoids for 6 days. Viabilities were determined by means of the trypan blue dye exclusion test. ○, acyclic retinoid; ●, 7-OH acyclic retinoid; △, all-*trans*-retinoic acid.

Table I. Values of 50% Growth-inhibitory Dose (ID_{50}), 50% Cytotoxic Dose (LD_{50}) and Ratio of LD_{50}/ID_{50} of Retinoids on PLC/PRF/5 Cells

	ID_{50} (μM)	LD_{50} (μM)	LD_{50}/ID_{50}
Acyclic retinoid	14	86	6.1
7-OH derivative of acyclic retinoid	26	61	2.4
All-*trans*-retinoic acid	28	40	1.4

retinoid up to day 6. Inhibition of cell growth was slight on treatment with acyclic retinoid from day 2 to day 6, the time corresponding to the logarithmic growth phase of PLC/PRF/5 cells. The doubling time in this phase

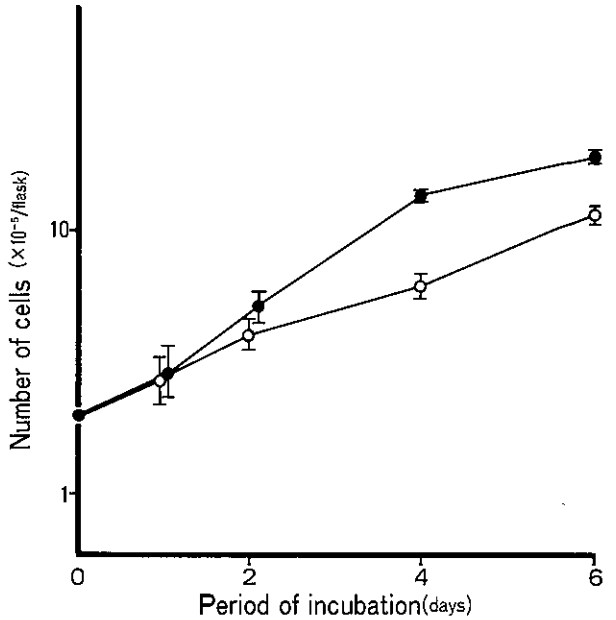

Fig. 3. Growth curves of PLC/PRF/5 cells incubated for 6 days in the presence (○) or absence (●) of 10 μM acyclic retinoid.

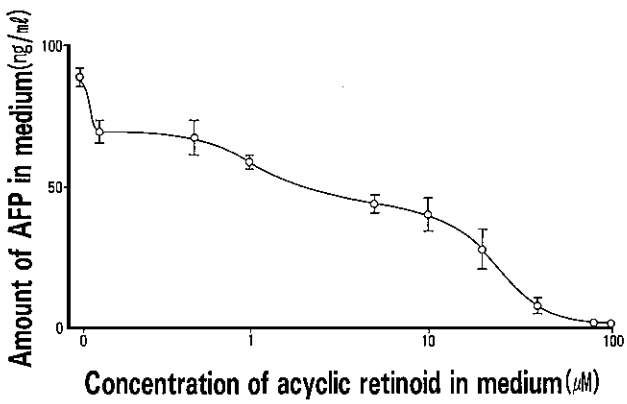

Fig. 4. Levels of α-fetoprotein in culture media of PLC/PRF/5 cells treated with acyclic retinoid for 6 days.

was 59.6±2.6 h for PLC/PRF/5 treated with acyclic retinoid, whereas it was 49.9±2.6 h for untreated cells ($P<0.05$). The evidence indicates that acyclic retinoid prolongs the cell cycle for PLC/PRF/5 cells.

Effect of acyclic retinoid on secretion of AFP PLC/PRF/5 cells constantly secrete AFP. The AFP level in culture medium was determined at 6 days after the start of treatment with acyclic retinoid by radioimmunoassay.

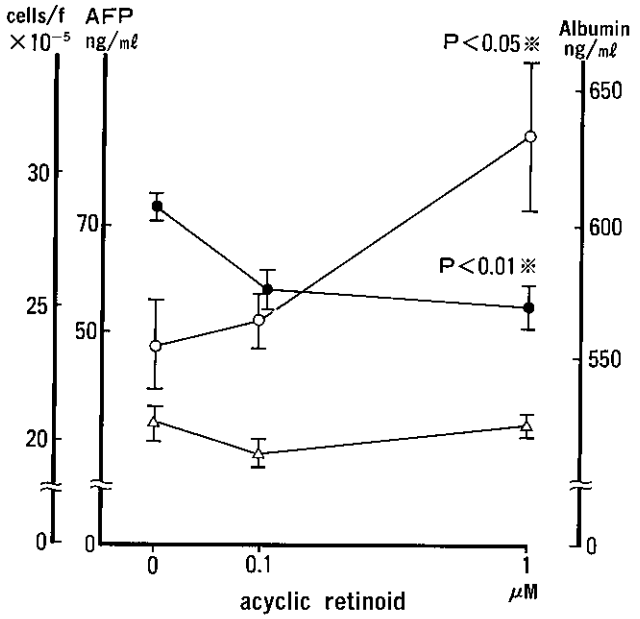

Fig. 5. Effects of acyclic retinoid on the α-fetoprotein (●) and albumin (○) levels in culture media and on the population (△) of PLC/PRF/5 cells.

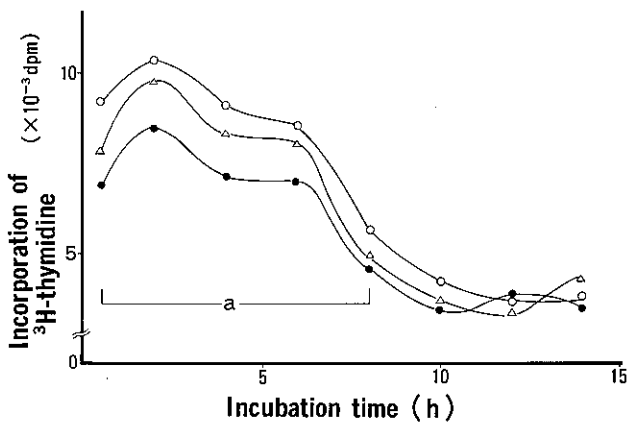

Fig. 6. Effects of acyclic retinoid on the incorporation of ^{3}H-thymidine by PLC/PRF/5 cells in S-phase synchronous culture. ○, Control; △, 1 μM acyclic retinoid; ●, 10 μM acyclic retinoid. a: In the whole period designated by "a", incorporation of ^{3}H-thymidine by the cells treated with 10 μM acyclic retinoid (●) was reduced, as compared to that of the control (○), with statistical significance at $P<0.01$.

Figure 4 shows dose-response curves for inhibition of AFP secretion by acyclic retinoid. Acyclic retinoid reduced secretion of AFP at as a low concentration as 0.1 μM and 50% inhibition was obtained at a concentration of 8.4 μM.

The biosynthesis of albumin is usually depressed in PLC/PRF/5 cells. However, the amount of albumin in the culture medium was significantly increased by acyclic retinoid dose-dependently. It is of great interest that the amounts of AFP and albumin were clearly changed in opposite directions by treatment with acyclic retinoid at a concentration of 1 μM, compared with those of the control (Fig. 5). At the concentrations in question, the number of cells did not change. Therefore we thought that acyclic retinoid acts directly on the regulation of protein synthesis, because it did not inhibit the synthesis of DNA in cells at concentrations of up to 1 μM, compared with the control. 7OH-acyclic retinoid and all-*trans*-retinoic acid could not be used in this experiment, because of the higher cytotoxicity of these compounds which suppress the cell growth before the secretion of AFP is affected.

Effects of acyclic retinoid on the incorporation of ^{3}H-thymidine by PLC/PRF/5 cells in S-phase synchronous culture When cells were incubated with 10 μM acyclic retinoid, incorporation of ^{3}H-thymidine by the cells was significantly reduced during a period from 0 to 8 h ($P<0.01$), while thereafter no significant difference was observed among control cells and cells treated with acyclic retinoid (Fig. 6).

DISCUSSION

Inhibitory effects of retinoids on hepatocarcinogenesis have been reported in animal experiments. Furthermore, Sasaki *et al.* demonstrated that acyclic retinoid inhibited secretion of AFP from human hepatoma cells (HuH-7) as well as cell growth.[7] Based on the previous results that acyclic retinoid inhibited tumor promotion on mouse skin, spontaneous hepatoma in mice and chemical hepatocarcinogenesis in rats, it is conceivable that acyclic retinoid could be developed as a preventive agent against human hepatoma. Therefore, we extended the study of acyclic retinoid to another cell line of human hepatoma, PLC/PRF/5 cells. As the ratio of ID_{50} (inhibition of cell growth) to LD_{50} (inhibition of cell viability) indicated, acyclic retinoid appeared to be less toxic to the cells than its hydroxy derivative or retinoic acid.

Growth-inhibitory effects of acyclic retinoid on cells were observed in the logarithmic phase, due to the prolonged doubling time of cells. Moreover, acyclic retinoid reduced incorporation of ^{3}H-thymidine in the S-phase of the cells, suggesting that acyclic retinoid regulated cell growth by affecting the *de novo* synthesis of DNA.

Acyclic retinoid inhibited secretion of AFP at a concentration of 0.1 μM and induced a reciprocal change

between the levels of AFP and albumin in the culture media. These results additionally suggested that acyclic retinoid might regulate the protein synthesis of AFP and albumin through nuclear/cytosol-retinoid binding proteins, which were recently discovered by several investigators.[10, 11]

Although the molecular biological studies of AFP and albumin are incomplete, the albumin gene is known to be located upstream of the AFP gene on the same genome.[12, 13] Therefore, acyclic retinoid, like retinoic acid, might be involved in the switching of gene expression for AFP and albumin.[14, 15] Further studies are under way in our laboratory to investigate the effects of acyclic retinoid on AFP and albumin mRNA levels in PLC/PRF/5 cells, and also to investigate the intracellular transport of the compound in the cytoplasm and in the nucleus, where acyclic retinoid is supposed to exert its effect(s) on DNA and/or RNA metabolism.

Acyclic retinoid seems to be one of the leading candidates for chemoprevention of hepatoma in the near future by virtue of its antitumor potential and relatively weak cytotoxicity on human hepatoma-derived cell line PLC/PRF/5.

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